BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/82, 5/10, A01H 5/00 — A1

(11) International Publication Number: **WO 98/13505**

(43) International Publication Date: 2 April 1998 (02.04.98)

(21) International Application Number: PCT/EP97/05217

(22) International Filing Date: 23 September 1997 (23.09.97)

(30) Priority Data:
A 1695/96 24 September 1996 (24.09.96) AT

(71) Applicant *(for all designated States except US)*: PLANT GENETIC SYSTEMS N.V. [BE/BE]; Jozef Plateaustraat 22, B-9000 Gent (BE).

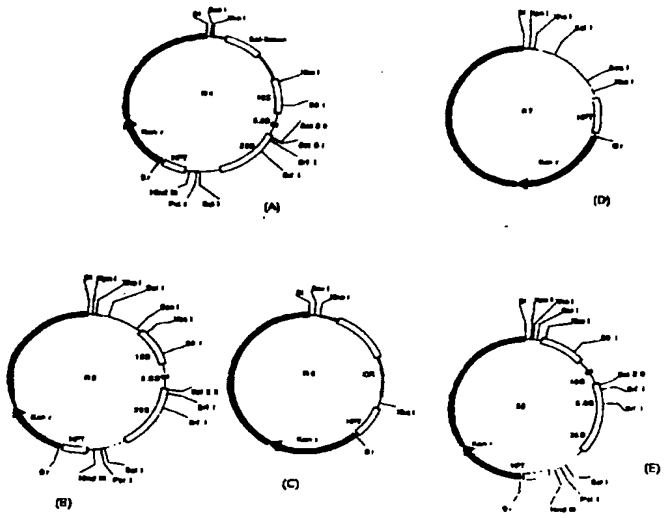
(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: BACHMAIR, Andreas [AT/AT]; Rennweg 14, A-1030 Vienna (AT). SCHWEIZER, Dieter [AT/AT]; Rennweg 14, A-1030 Vienna (AT).

(74) Agents: SONN, Helmut et al.; Riemergasse 14, A-1010 Vienna (AT).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: DNA-CONSTRUCTS COMPRISING INTERGENIC RIBOSOMAL DNA AND METHODS TO PRODUCE PROTEINS USING THESE DNA-CONSTRUCTS



(57) Abstract

Provided are a DNA-construct, comprising the following fragments: ribosomal DNA, promoter region and heterologous coding region, a method for the production of proteins and for enhancing copy number or expression using these DNA-constructs, as well as host-organisms, particularly plants, comprising these constructs.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

DNA-CONSTRUCTS COMPRISING INTERGENIC RIBOSOMAL DNA AND METHODS TO PRODUCE PROTEINS USING THESE DNA-CONSTRUCTS

The present invention provides DNA-constructs comprising DNA fragments of the intergenic spacer between ribosomal rRNA gene repeats, as well as methods to produce proteins and methods for enhancing copy number or expression using these DNA-constructs, in eukaryotic cells, particularly in plant cells.

BACKGROUND

An important part of the genome of higher eukaryotic organisms consists in ribosomal DNA. Numerous transcription units (about 600 in the case of Arabidopsis thaliana) are arranged one directly after the other, in tandem. Cytogenetically these units form the nucleolus-organizing region and they are located near a telomere of a restricted number of chromosomes (e.g. chromosomes 2 and 4 from Arabidopsis thaliana). The RNA-polymerase I is specifically responsible for the transcription of ribosomal RNA (rRNA)-genes. Due to their high copy number, the ribosomal RNA-genes were the first genes to be analysed molecularly. The repetitive nature of these genes has hampered considerably their further analysis.

Progress in the understanding of the transcription of ribosomal DNA was principally obtained using in vitro systems obtained from animal cells. In plants, the (intergenic) region between transcriptional units from many genera has been investigated, but functional interpretations are largely based on sequence comparisons.

The control elements for transcription are located in the intergenic region (IGR), also called intergenic spacer (IGS). The IGR is the DNA region located between the 25S RNA encoding region of the preceding rRNA gene unit and the 18S RNA encoding region of the following rRNA gene unit. It consists in general of a 3' external transcribed spacer extending from the end of the region encoding the mature 25S RNA up to the transcription termination site of the preceding rRNA gene unit, and a 5' external transcribed spacer extending from the transcription initiation site up to the beginning of the region encoding the mature 18S RNA of the next rRNA gene unit, with in between these two regions a non-transcribed spacer. In higher plants, the IGR contains repetitive sequence motifs in the majority of the cases. The exact base sequence of these motifs is probably under low selective pressure, since there is mostly little or no sequence similarity between related species. This is in contrast with the good conservation of another part of the IGR, namely the region surrounding the transcription start.

Considerable progress in the understanding of ribosomal transcription was recently made, using a transient expression system in Arabidopsis thaliana (Doellling, J.H. and Pikaard, C.S. (1995) "The minimal ribosomal RNA gene promoter of Arabidopsis thaliana includes a critical element at the transcription initiation site, Plant J. 8, 683-692; Doellling, J.H., Gaudino, R.J. and Pikaard, C.S. (1993) "Functional analysis of Arabidopsis thaliana ribosomal RNA gene and spacer promoters in vivo and by transient expression", Proc. Natl. Acad. Sci. USA, 90, 7528-7532). Further, in vitro transcription systems are available for beans and tobacco (Yamashita, J., Nakajima, T., Tanifuji, 5. and Kato, A.. (1993) "Accurate transcription initiation of Vicia faba rDNA in a whole cell extract from embryonic axes", Plant J. 3, 187-190; Fan, H. Yakura, K., Miyanishi, M., Sugita, M. and Sugiura, M. (1995) "In vitro transcription of plant RNA polymerasel-dependent rRNA genes is species-specific" Plant J., 8, 295-298).

SUMMARY OF THE INVENTION

In accordance with the invention, DNA constructs are provided, comprising the following operably linked DNA fragments:

- a DNA fragment comprising a ribosomal DNA sequence preferably derived from a plant, preferably derived from the intergenic region of the ribosomal DNA of a plant; particularly comprising the upstream SalI repeats from the intergenic region from the ribosomal DNA of Arabidopsis thaliana or a similar region from another plant;
- a fragment comprising an expressible promoter region, especially a plant-expressible promoter region, preferably a promoter recognized by RNA polymerase II;
- a heterologous coding region; and optionally
- a transcription termination and polyadenylation region, preferably a region which is active in plant cells.

Particularly preferred ribosomal DNA sequences comprise a DNA sequence selected from the DNA sequence of SEQ ID N° 1 from nucleotide position 486 to 5212, the DNA sequence of SEQ ID N° 1 from nucleotide position 1263 to nucleotide position 3003, the DNA sequence of SEQ ID N° 1 from nucleotide position 569 to nucleotide position 2862, the DNA sequence of SEQ ID N° 1 from nucleotide position 1263 to nucleotide position 2862, the DNA sequence of SEQ ID N° 1 from nucleotide position 486 to 5212, the DNA sequence of SEQ ID N° 1 from nucleotide position or 596 to 5373.

Also provided are a method to produce proteins, comprising the following steps:

- introducing a DNA-construct according to the invention in a suitable host organism;
- cultivating the host-organism under conditions which allow expression of the protein encoded by the structural gene; and

- harvesting the expressed protein.

In addition, a method for enhancing the stability, the copy number or the expression of a transgene in a plant is provided comprising the following steps:
- introducing a DNA construct according to the invention in a plant cell; and
- regenerating a plant from the transformed plant cell.

Also in accordance with the invention, host-organisms, particularly plants and plant cells comprising the DNA-constructs according to the invention, integrated in their nuclear genome, are provided.

BRIEF DESCRIPTION OF THE FIGURES

Fig 1:	the restriction map of the ribosomal DNA from Arabidopsis thaliana;
Fig 2:	(A) the sequence of the V1 region of A. thaliana 25S rRNA before (left) and after (right) introduction of the insertion sequence (B) the sequence of the oligonucleotide used for the detection of 25S rRNA;
Fig 3:	Analysis of the integration of the transgene;
Fig 4:	Primer extension analysis to detect the transgenic rRNA;
Fig 5:	determination of the 5'end of A. thaliana 25S rRNA;
Fig 6:	serial silver-stained sections through cells with trangene R4
Fig 7:	analysis of transcription of the ectopic ribosomal gene;
Fig 8(a)-(e):	binary vectors R4 to R8.
Fig 9-A/B:	schematic representation of the binary vectors comprising respectively lacking the upstream SalI repeats in front of a chimeric CaMV35S-gus gene

DETAILED DESCRIPTION

In one aspect of the present invention, a DNA-construct is provided which allows improved expression of foreign proteins in eukaryotic cells, particularly in plant cells. A DNA-construct according to the invention comprises in reading direction, the following operably linked DNA fragments:
- a DNA fragment comprising a ribosomal DNA sequence, preferably derived from a plant
- a fragment comprising an expressible promoter region, especially a plant-expressible

promoter region,
- a heterologous coding region; and optionally
- a transcription termination and polyadenylation region, preferably a region which is active in plant cells.

Surprisingly with such a construct a higher number of transformants, a higher copy number of the transgene, as well as an enhanced stability of the transgene and enhanced expression can be obtained. This is even more unexpected since the skilled artisan would not have expected to obtain a more efficient expression system by combining a DNA fragment with a role in the recognition and/or transcription initiation by RNA-polymerase I (the ribosomal DNA) with a DNA-fragment which is normally transcribed by RNA-polymerase II.

Although the promoter region in the recombinant DNA according to the invention is preferably a promoter recognized by RNA polymerase II, the promoter may also be comprised within the ribosomal DNA, which could make the construction of the recombinant DNA according to the invention easier. As used herein, the term "plant-expressible promoter region" means a promoter which is capable of driving transcription in a plant cell. This includes any promoter of plant origin, but also any promoter of non-plant origin which is capable of directing transcription in a plant cell, i.e., certain promoters of viral or bacterial origin such as the CaMV35S or the T-DNA gene promoters, as well as promoter regions derived from bacteriophages and recognized by the specific single subunit RNA polymerases, such as T7 or T3 RNA polymerase specific promoters. In the latter case, it is imperative that the host cells comprise functional RNA polymerase recognizing the specific promoters.

It goes without saying that when it is mentioned that the ribosomal DNA should be derived from a plant, it is meant that the sequence of that ribosomal DNA fragment should be identical or similar to the sequence as it is found in a plant. Of course, the DNA fragment can have been cloned in an intermediate organism, such as E. coli or be completely or partially synthetic.

Ribosomal DNA fragments suitable for the invention are capable to direct the chromatin structure in such a way that the DNA constructs according to the invention, integrated in the nuclear genome are located in the neighbourhood of the nucleolus or that the genomic region wherein the transgene is integrated, adopts a similar chromatin structure as found in the nucleolus. Methods to determine the spatial location of a DNA fragment in a cell are known to the person skilled in the art, and one such method is set forth in detail in the Examples. Mehods to determine the structural characteristics of chromatin,

particularly the degree of accessibility of the DNA for interacting molecules, are also know in the art (e.g. by determining the accesibility to micrococcal nuclease or by crosslinkage of chromosomal proteins (Ausubel et al. (1994) see below, Dammann et al. (1995) "Transcription in yeast rRNA gene locus: Distribution of the active flanking regulatory sequences", Mol. Cell. Biol. 15, 5294-5303).

In a preferred embodiment, the ribosomal DNA is specifically adapted to the host-organism wherein the recombinant DNA according to the invention will be introduced; eg. a ribosomal DNA derived from that host organism or from a closely related species.

The ribosomal DNA is preferentially derived from the intergenic region of the ribosomal DNA. In the case of Arabidopsis thaliana (where the intergenic region from variety Col0 has the DNA sequence of SEQ ID N° 1 from nucleotide position 486 to 5212), especially preferred are DNA fragments comprising the "upstream SalI repeats" from the intergenic region of Arabidopsis thaliana ribosomal DNA. These upstream SalI repeats are organized in three blocks [the so-called SalI box 1 (SEQ ID N° 1 from nucleotide position 1263 to 1557) SalI box 2 (SEQ ID N° 1 from nucleotide position 1883 to 2177) and SalI box 3 (SEQ ID N° 1 from nucleotide position 2503 to 3003)] and it is thought that inclusion of a DNA fragment comprising these SalI repeats (such as a fragment having the DNA sequence of SEQ ID N° 1 from nucleotide position 1263 to nucleotide position 3003) is sufficient to obtain the effects according to the invention. In fact, the presence of a complete third SalI repeat is not required, since a fragment comprising only the part of SalI box 3 up to the EcoRI site (having the DNA sequence of SEQ ID N° 1 from nucleotide position 569 to nucleotide position 2862) can be used to similar effect. Thus, in a preferred embodiment, the ribosomal DNA comprises a fragment having the DNA sequence of SEQ ID N° 1 from nucleotide position 1263 to nucleotide position 2862. Furthermore, larger fragments comprising the upstream SalI repeats can be used, such as a fragment comprising the complete intergenic region (having the DNA sequence of SEQ ID N° 1 from nucleotide position 486 to 5212) or even a fragment comprising parts of the regions coding for the rRNA transcripts, particularly the 18S transcript (such as a fragment having the DNA sequence of SEQ ID N° 1 from nucleotide position 596 to 5373). In addition it is possible to use a fragment comprising a complete ribosomal gene unit (the DNA sequence of which can be obtained by merging the overlapping DNA sequences available from the EMBL database under the Accession numbers X16077, X52320 and X15550) although when using such DNA constructs, the effects as described in this invention might be less pronounced, possibly due to shielding effects.

For other host plants, particularly suited DNA fragments comprise those domains of the ribosomal DNA which correspond to these domains in Arabidopsis, particularly those

which are derived therefrom. Preferred are DNA fragments comprising the intergenic spacers, located between the DNA regions coding for the 25S and 18S regions, particularly DNA fragments comprising the non-transcribed intergenic spacers located between the transcription termination site of the previous rRNA gene unit and the transcription initiation site of the following rRNA gene unit. Other rRNA intergenic spacers are known in the art at least for corn [McMullen et al., Nucl. Acids Res. 14: 4953-4968 (1986)Toloczyki and Feix, Nucl. Acids Res 14:4969-4986 (1986)] rye [Appels et al, Can J Genet Cytol 28:673-685 (1986)], wheat [Barker et al, J. Mol. Biol. 201: 1-17 (1988)], radish [Delcasso-Tremousaygue et al., Eur. J. Biochem 172: 767-776 (1988)], rice [Takaiwa et al., Plant Mol. Biol. 15: 933-935(1990)], mung bean [Gerstner et al, Genome 30: 723-733 (1988), Schiebel et al., Mol Gen Genet 218: 302-307 (1989)], potato [Borisjuk and Hemleben,Plant Mol Biol. 21; 381-384 (1993)], tomato [Schmidt-Puchta et al., Plant Mol Biol 13: 251-253 (1989)], Vicia faba [Kato et al, Plant Mol. Biol. 14: 983-993 (1990)], Pisum sativum [Kato et al., supra (1990)] and Hordeum bulbosum [Procunier et al., Plant Mol Biol. 15: 661-663 (1990)]. Moreover intergenic spacers from a plant can be straightforwardly amplified in a PCR reaction using oligonucleotides corresponding to the 3' end of the conserved 25S mature rRNA encoding region and the 5' end of the conserved 18S mature rRNA encoding region.

It has been reported (Gruendler et al., 1991, J. Mol. Biol. 221, 1209-1222) that the number of upstream SalI repeats may differ in different varieties or isolates of the same species (in casu A. thaliana) . Such variants are also encompassed by the invention.

It is also possible to use a modified ribosomal DNA, which is derived from the original ribosomal DNA by mutation, insertion or deletion, as long as the essential functional, particularly related (e.g. with DNA-binding proteins such as polymerases) or at least the essential topological characteristics from the ribosomal DNA are not lost by the modification.

As used herein "coding region" or "coding sequence" refers to a DNA region which when provided with appropriate regulatory regions, particularly a promoter region, is transcribed into an RNA which is biologically active i.e., which is either capable of interaction with another nucleic acid such as e.g. an antisense RNA or a ribozyme or which is capable of being translated into a biologically active polypeptide or protein.

As used herein, the term "heterologous" with regard to a coding region refers to any coding region which is different from the rRNA coding region naturally associated with ribosomal DNA fragment used in the chimeric DNA according to the invention.

As coding region, all known natural or modified coding regions can be used, which are compatible with the host-organism, in other words that the expressed product is expressed in such a way that the product is not too toxic for the host, particularly that it leads to a significant expression, before the product is collected, if collected at all. Particularly, the coding region might encode a vaccine, an antibody, a therapeutical protein, an insecticidal protein such as a Bt toxin or the minimal toxic fragment thereof, a protein used in food technology, an antisense-RNA or a ribozyme.

The DNA-construct can be made in such a way that it is adapted to a particular host or transformation system. In a preferred embodiment, the DNA construct according to the invention is in the form of a vector. Particularly, the DNA construct is a T-DNA vector.

Although the ribosomal DNA fragment preferably precedes the promoter region and the heterologous coding region in the DNA-constructs according to the invention, it is expected that similar effects will be achieved when the ribosomal DNA fragment is placed downstream of the operably linked promoter region and coding region.

In another aspect, the present invention also provides a process for the production of proteins comprising the following steps:

- introducing a DNA-construct according to the invention in a suitable host organism,
- cultivating the host-organism under conditions which allow expression of the protein encoded by the structural gene
- optionally, harvesting the expressed protein.

In yet another aspect, the present invention provides a process for enhancing the stability, the copy number and/or the expression of a transgene, especially in a plant, comprising the following steps:

- introducing a DNA construct according to the invention in a cell, preferably in a plant cell
- regenerating an organism, preferably a plant, from the transformed cell

The method of the invention is particularly suited to enhance the stability and expression of recombinant genes which have at least partially homology to a sequence present in the host cell, particularly the plant cell, such as transgenes in multiple copies or different transgenes comprising similar sequences such as e.g. the same promoter. It is known that such transgenes are frequently prone to homologous recombination, as well as to reduction in expression by e.g. methylation, or co-suppression events. Although not intending to limit the invention to a mode of action, it is thought that the localization of the transgenes resulting from the transformation of

the DNA constructs according to the invention, in or in the neighbourhood of the nucleolus or the enforcement of a nucleolus-like chromatin structure on the chromosomal region wherein the transgene is integrated, reduces the recombination between homologous sequences (thus increasing stability), the methylation and any other events resulting in a reduction in expression of such transgenes.

Introduction of the DNA can be done using any method or manner and different techniques - dependent upon the host-organism used - are available to the skilled artisan. Particularly preferred ways of introduction of DNA are T-DNA transformation, electroporation or particle -bombardment as well as plasmid- or virus-transformation.

The optimal cultivation conditions depend on the used host-organism and on the nature of the expressed protein. These conditions are again known to the person skilled in the art (for well-known host-organism) and/or can be easily determined or optimised using known biotechnological protocols.

The harvest of the proteins is also preferably performed according to standard methods for the concerned host-organism and depends also on the way the protein is expressed (e.g. whether it is secreted or excreted in the culture medium, or accumulates inside the host-organism, or is included in specific compartments, ...). As with the cultivation step, also here the person skilled in the art can without undue experimentation determine and or optimize the harvesting conditions for a particular host/structural protein system.

Preferred host-organisms, particularly plants are well-known and well defined systems, such as Arabidopsis thaliana, or economically important crops such as tobacco, corn, wheat, potato, rice, soy beans, barley, rye, a brassica vegetable, a Beta species or manioc.

Also provided by the present invention is a host-organism comprising the DNA-construct of the invention. Preferably, this host-organism is a eukaryotic cell, particularly a plant cell, as well as an organism comprising these cells or generated from these cells.

In yet another aspect the invention provides reproduction material, particularly plant reproduction material, comprising cells which comprise the DNA-construct according to the invention.

The invention and advantages thereof are further illustrated in the examples and Figures which are in no way limitative.

In the examples and in the description of the invention, reference is made to the following sequences of the Sequence Listing:

SEQ ID No.1:	DNA sequence of the intergenic region of rRNA gene repeats of A. thaliana
SEQ ID No.2:	oligonucleotide
SEQ ID No.3:	oligonucleotide
SEQ ID No.4:	oligonucleotide E
SEQ ID No.5:	oligonucleotide Q
SEQ ID No.6:	DNA sequence of the A. thaliana region between the 3' end of 5.8S rDNA and the 5' end of the 25S rDNA

EXPERIMENTAL

Unless stated otherwise in the Examples, all recombinant DNA techniques are carried out according to standard protocols as described in Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, NY and in Volumes 1 and 2 of Ausubel et al. (1994) Current Protocols in Molecular Biology, Current Protocols, USA. Standard materials and methods for plant molecular work are described in Plant Molecular Biology Labfax (1993) by R.D.D. Croy, jointly published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications, UK. These publications also include lists explaining the current abbreviations.

1. Vector construction

A SalI fragment from lambda-phage ATR3 (Grundler, P., Unfried, I., Pointner, R. and Schweizer, D. (1989) "Nucleotide sequence of the 25S-18S ribosomal gene spacer from Arabidopsis thaliana", Nucleic Acids Res., 17, 6395-6396; Unfried, I., Stocker, U. and Gründler, P. (1989) "Nucleotide sequence of the 18S rRNA gene from Arabidopsis thaliana Col-0", Nucleic Acids Res., 17, 7513; Unfried, I. and Gründler, P. (1990) "Nucleotide sequence of the 5.8S and 25S rRNA genes and of the internal transcribed spacers from Arabidopsis thaliana", Nucleic Acids Res., 18, 4011), which extends from the vector-insert border (nucleotide position 1400 from sequence X16077) to the SalI-restriction site in position 1263 of the sequence X15550, was inserted in the SalI restriction site of the binary vector pBIB Hyg (Becker, D. (1990) "Binary vectors which allow the exchange of plant selectable markers and reporter genes", Nucleic Acids Res., 18, 203). The orientation of the insert was such that the 18S-gene is located near the

Asp718 restriction site. The resulting vector, R2, is cleaved with Asp718, treated with Klenow-enzyme and cleaved partially with SfiI. A fragment from ATR3, which extends from the (blunted) XhoI-restriction site (position 569 in sequence X15550) to the SfiI restriction site (position 1490 in sequence X16077), is inserted and completes the sequence of a ribosomal transcription unit in vector R3.

To insert an oligonucleotide, a fragment from the SalI-restriction site in position 305 from sequence X 16077 to the PstI restriction site in position 1425 from X52320, comprising the largest part of the 18S rDNA-sequence and the 5'-end of the 25S rDNA sequence was first cloned in pSK+ (plasmid pBlu/SP). After cleaving with XbaI and SrfI , filling in of the sticky ends with Klenow-enzyme and ligation, the ligation product is treated with XhoI and EcoNI, treated with Klenow enzyme and religated. The resulting vector now has a single AatII restriction site. The oligonucleotide CCAAGGTAACCTTCGACGT (SEQ ID N° 2) and CGAAGGTTACCTTGGACGT (SEQ ID N° 3) were allowed to anneal and were inserted in the AatII-restriction site. After checking the sequence, a BamHI/BstBI fragment was transferred to the vector pBlu/SP (vector pBlu/SP+E). An SfiI/SrfI fragment from vector pBlu/SP+E was inserted in vector R3 (vector R4; Fig. 8a).

2. Plant transformation

Plant transformation was performed as described by Valvekens, D., van Montagu, M. and van Lijsebettens, M. (1988) "Agrobacterium tumefaciens-mediated transformation of Arabidopsis thaliana root explants by using kanamycin selection", (Proc. Natl. Acad. Sci. USA, 85, 5536-5540), except that hygromycin selection was used. The selectable marker in the plasmids used in the examples 1 and 2 was indeed hygromycin phosphotransferase.

3. DNA-isolation

DNA-isolation was performed as described (Dellaporta, S.L., Wood, J. and Hicks, J.B. (1983) "A plant DNA minipreparation version II", Plant Mol. Biol. Rep., 1, 19-21), with the following modifications: After phenol extraction, 1/10 of the volume ethidiumbromide (10g/l) is added to the DNA solution as well as CsCl (0.9 g/ml). The solution is kept on ice for 30 min. After centrifugation (5 min 5000 RPM) the clear solution is adjusted with CsCl to a density of 1.6 g/ml and centrifuged for 3 hours at 80 000 rpm in a Beckman NVT90 rotor. DNA is harvested using standard methods from the gradient (Ausubel, F.M., Brent, R., Kingston, R.F., Moore, D.O., Seidman, J.G., Smith, J.A. and Struhl, K. (Eds.) (1987) Current Protocols in Molecular Biology, John Wiley & Sons, Inc., New York).

4. DNA-analysis through gel-blot

Purified DNA from callus material was digested with restriction enzymes such as BstEII and used for gel-blot-analysis (Ausubel et al., 1987).

5. RNA preparation

RNA was prepared as described in Logemann, J., Schell, J and Willmitzer, L. ((1987) "Improved method for the isolation of RNA from plant tissues" Anal. Biochem., 163, 16-20) with the following modifications: callus material was ground in liquid nitrogen and mixed with 2 ml extraction buffer (8M guanidium chloride, 20 mM MES, 20 mM EDTA, 50 mM 2-mercaptoethanol, 0.5 % Sarcosyl pH about 3) per gram callus material. After thawing the suspension was transferred to a centrifuge tube, and extracted with one volume phenol/chloroform/isoamyl alcohol (50:49:1). After heavy vortexing, the mixture is kept on ice for about 15 minutes and centrifuged at 10000g for about 10 min. The aqueous phase is transferred to a new tube, and 1/10 volume sodium acetate (1M) and 1 volume of ethanol is added, to precipitate the nucleic acids. After mixing the solution it was centrifuged at 10000g. The pellet was washed with 3M sodium acetate and 70 % ethanol. After evaporation of the residual alcohol, the pellet is dissolved in water through incubation at 60 to 65°C for about 3 hrs.

6. Primer extension analysis

25 ng of oligonucleotide E (GAAGACGTCGAAGGTTACCTTGG (SEQ ID N° 4); an oligonucleotide which exclusively binds to the rRNA with the 19 nucleotide insertion) or Q (CCCGGTTCGCTCGCCGTTACTAAG (SEQ ID N° 5) ; nucleotide 950 to 927 of the non-coding strand of sequence X52320, which can bind to all 25S RNA-molecules of A. thaliana) were phosphorylated with 10 μCi^{32} P-gamma-ATP in a volume of 10 μl for 90 minutes, extracted with phenol/chloroform after addition of 1 mg of tRNA and precipitated with sodium-acetate and ethanol. The oligonucleotide was incubated with 2.5 μg of total RNA from A. thaliana in 11 μl water to allow hybridization, and cooled from 90°C to 63 °C and then to 52°C in a time span of about one and a half hour. For the extension reaction, 5 μl 5xRT buffer (provided by the enzyme manufacturer), 2μl dNTPs (2mM) and 0.5 μl (12 units) AMV reverse transcriptase (Boehringer Mannheim) were added. After one hour incubation at 38°C, 1 μl DNAse free RNAse A (5 mg/ml; Boehringer Mannheim) were added, and the mixture was incubated further for 30 min at 37°C. The reaction mixture was separated on a denaturing polyacrylamide gel as used for DNA-sequencing.

7. Sectioning and staining of cells

Callus material was fixed with 2.5% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer and embedded in LR White Harz. Serially following sections with a thickness of 800 nm (resulting in about 5 to 6 sections per nucleus) were produced using a Reichert Ultracut E Microtome. The sections were stained with Richardson's Dye and analysed using a Leitz Dialux Microscope.

EXAMPLES

Example 1 Introduction of the DNA-constructs in A. thaliana and analysis of transformed cells.

Sequence comparison of the ribosomal RNA from different species revealed the presence of several regions with high variability. In many instances, the variable region has a hairpin structure which may be either longer, shorter or even completely absent. One such variable region in the large rRNA, named V1, was used, to insert a specific nucleic acid sequence. This region is also close enough to the beginning of the mature 25S rRNA so that extension experiments can be performed.

A DNA fragment of the rDNA which comprises somewhat more than one repeat unit (transcription unit) was inserted into the binary vector pBIB Hyg (Becker, D., 1990). The construct contains the presumed signals for transcription termination on both ends of the repeat unit. The structure of this construct (named R4) and the restriction map of the ribosomal repeat unit of A. thaliana are represented in Fig. 1 and Fig. 8(a).

The open boxes indicated 18S, 5.8S and 25S represent the regions coding for the three ribosomal RNAs. "Upstream Sal repeats" indicates three blocks of repetitive DNA in the intergenic region, which comprise a lot of SalI restriction sites. Bold lines at both ends of the scheme indicate the vector DNA. The BstEII restriction site, introduced by the nucleotide-insertion is indicated with "B". Vector R5 is different from vector R4 in the absence of "the upstream SalI repeats", as well as in the presence of the KpnI restriction site at the border of the ribosomal unit (K). B, BstEII; E, EcoRI; F, FspI; H, HindIII; K, KpnI;P, PstI; Pc, PacI; S, SalI; Sc, ScaI; Sf, SfiI; Sr, SrfI; X, XbaI; Xh, XhoI.

The nucleic acid sequence of the region is available in the EMBL-database under accession

number X16077, X52320 and X15550. Variants of a part of the ribosomal DNA are available in the EMBL-database under accession number X52631, X52637 and X52636 (Gründler et al., 1989; Unfried et al., 1989; Unfried et al., 1990; Gründler, P., Unfrie, I., Pascher, K. and Schweizer, D. (1991) rDNA intergenic region from Arabidopsis thaliana: structural analysis, interspecific variation and functional implications. J. Mol. Biol., 221, 1209-1222).

As mentioned above, a ribosomal gene-unit (Fig. 1) was marked with a small nucleotide insertion in the large (25S) ribosomal RNA to be able to recognize it specifically and discriminate it from other ribosomal gene-units present in the genome. Fig. 2 shows the nucleotide insertion as well as an oligonucleotide which allows detection of transcription of this (marked) ribosomal gene-unit. The insertion sequence has a BstEII restriction site, which can be used for the specific recognition of the gene-unit (a), sequence of the region V1 of A. thaliana 25S rRNA before (left) and after (right) insertion of the oligonucleotide sequence. The secondary structure of the insertion is hypothetical and is only for the purpose of comparison with the structure without insertion. The insertion has a BstEII-restriction site in the DNA. (b), Sequence of the oligonucleotide allowing detection and quantification of 25S ribosomal RNA through reverse transcription.

A further construct, named R5 (see Fig. 8(b)), differs from R4 in that the "upstream SalI repeats" have been removed.

The constructs R4 and R5 were introduced in root explants of A. thaliana plants using Agrobacterium tumefaciens. Adjacent to the ribosomal unit a gene is located which is transcribed by RNA-polymerase II and confers resistance to hygromycin B (hygromycin-phosphotranferase-gene; Becker, D., 1990). Transformed cells/calli are selected by their resistance to hygromycin. DNA from these calli is used for DNA-blot-experiments.

Fig 3 shows that the ribosomal transgene-copies and the adjacent marker gene can integrate in different places in the genome. For digestion of the genomic DNA the enzyme BstEII was used which does not cleave in the ribosomal genes of the A. thaliana ecotype Col-0. Therefore, the ribosomal DNA is visible as a high-molecular weight band or is even partially retained in the gel-slot. On the contrary, ribosomal transgenes which have not integrated between natural ribosomal DNA repeats can give rise to smaller bands, since in the genomic region near the integration place, BstEII restriction sites should be present in a statistically random way. Exactly the latter result can be seen in the analysed calli.

The DNA-hybridization experiments shown in Fig. 3 demonstrate that integration of the transgene at least in the majority of cases does not occur in the regions of the ribosomal DNA. DNA from callus material was restricted with BstEII and transferred to a nylon-membrane after size fractionation on an agarose gel. Lanes 1 and 2 contain DNA from non-transformed callus material, and from callus material transformed by DNA construct R4 respectively. DNA from the hygromycin phosphotransferase gene was used as probe. The detected fragments have one side the BstEII restriction site located in the 25S rRNA transgene and on the other hand a BstEII restriction site in the DNA of the integration place. Since the endogenous rDNA does not contain BstEII restriction sites, the presence of such restriction sites in the host-DNA in the immediate neighbourhood of the trangenes, constitutes proof for integration of the transgene outside of the ribosomal DNA. Lanes 3 and 4 show the same digested DNA as in lanes 1 and 2, but here ribosomal DNA was used as probe. The DNA from the calli remains either in the gel-slot, or moves as high-molecular DNA at the limits of the separating power of the gel. Lanes 5-8 contain DNA from three further independently transformed calli, whereby lane 8 has been hybridized with a ribosomal probe.

From the transformed calli, RNA was prepared and used for the primer extension analysis. Therefore, an oligonucleotide was designed, which binds to the insertion in the variable region V1, but not to RNA which does not contain such an insertion. The synthetic DNA-fragment indicated as oligonucleotide E in Fig 2 was particularly suited. A reverse transcript using this oligonucleotide as primer, was obtained in transformed calli, but not in non-transformed calli. The activity of the ribosomal transgene can be detected in the majority of cases (Fig. 4):

With oligonucleotide E as primer, a reverse transcription reaction yields a product of about 150 bases (indicated by asterisk). Several calli were pooled to prepare RNA ((a) Lane 1: callus material not transformed with ribosomal transgene DNA; lane 2: callus material transformed by the ribosomal transgene R4; (b) gel slot (indicated by arrow) and size marker ; precursors with higher molecular weight (2 or 3 asterisks) can be seen in a lot of experiments; lane 1: size marker; lane 2, as lane 1 in (a); lane 3: as lane 2 in (a)).

As follows from the mode of selection, the adjacent hygromycin resistance gene is active in all cases.

The processing of ribosomal transgenes is comparable to the natural ribosomal gene, in that the same 5'end of the 25S rRNA could be determined (Fig 5): the 5'end of the normal genomic 25S rRNA was determined using oligonucleotide Q, the 5'end of the transgene marked with the inserted sequence was determined using oligonucleotide E. A comparison

with the sequencing ladder proves identical ends. Moreover, the end of the RNAs is different than presumed up to now, in that the 25S rRNA is somewhat longer than accepted until now (Fig. 5). Also, processing intermediates of the 25S rRNA can be detected, indicating again a similar processing of the unchanged rRNA and the rRNA with the inserted sequence.

The determination of the 5'end of the 25S rRNA from A. thaliana is presented in Fig. 5. Fig 5 (a) shows the primer extension analysis with oligonucleotide Q, which can bind all 25S rRNA transcripts. On the right side a sequencing ladder obtained using the same oligonucleotide as primer is represented. Fig 5(b) shows the reverse transcription with oligonucleotide E, which is specific for the transgenic ribosomal copy. On the left side a sequencing ladder obtained using the same oligonucleotide as primer is represented. The asterisk marks the final base of the reverse transcripts. Transgenic and endogenous ribosomal 25S rRNA have an identical 5'-end Fig 5(c) is analogous to lane 3 in Fig 4(b), but with sequencing ladder. Fig 5(d) (and SEQ ID N° 6) shows the sequence of the ribosomal DNA with indication of the 5'ends of the 25S rRNA, as well from a precursor (one asterisk and two asterisks respectively). The dot represents the previously presumed 5'end. Bold font indicates the part present in 25S, respectively 5.8S RNA.

The identical processing of transgenic and natural ribosomal RNA indicates that the components necessary for such a processing (which normally occur in the nucleolus) have access to the ectopically integrated transgene. It was further analysed, whether the latter one could be located in a nucleolus or in the neighbourhood of a nucleolus. A positive result would correspond to the situation found in Drosophila (Karpen, G.H., Schaefer, J.E. and Laird, C. D. (1988) "A Drosophila rRNA gene located in euchromatin is active in transcription and nucleolus formation", Genes Dev. 2, 1745-1763), a negative result would be similar to the situation found in baker's yeast. Transgenic Arabidopsis nuclei were stained with silver and serial thin sections were made. Fig 6 and Table 1 show that Arabidopsis cells with the mentioned transgene have on average one additional nucleolus.

Table 1: Average number of nucleoli in callus cells transformed with R4, and in non-transformed control cells (in each case, 25 cells were analysed by serial sectioning as in Fig 6)

	Average number of nucleoli
R4 transgenic cells	2.8 ± 0.5
Control cells	2.0 ± 0.3

These serial sections through cells with transgene R4 and silver staining show the presence of additional nucleoli ((a) through (j): Sections through A. thaliana callus-cells without ectopic ribosomal DNA; (k) through (t), as (a) through (j), but with callus material comprising ectopic rDNA (remarkable cells are indicated by asterisks); in (k) through (t) additional nucleoli can be seen.

The selected callus-nuclei have on average three nucleoli, while this number in non-transformed callus material is around two; The size of the three nucleoli cannot be distinguished in such a way as to state that one of them contains only the ectopic copies. It can equally be assumed that the available active ribosomal genes are divided between all three copies. Further experiments indicated that the gene coding for hygromycin resistance is located in a position at least in the neighbourhood of the nucleoli.

In a further series of experiments, it was analysed whether the construct R5, lacking the upstream SalI repeats, was also transcribed, and whether the transcription level can be distinguished from the transcription level of construct R4.

Therefore, a reverse transcription and analysis was performed on plant material which was either transformed only with vector sequences (lanes 1 in both panels of Fig 7), with ribosomal copies lacking the upstream SalI repeats (lanes 2 in both panels) or with the complete ribosomal copies (Lanes 3 in both panels). In Fig 7 (a) the oligonucleotide E (specific for the transgenic copy) was used; in Fig 7(b) oligonucleotide Q binding to all 25S rRNA molecules was used (as a control for similar amounts of RNA).

Fig. 7 shows that no difference could be demonstrated. To exclude positional effects on some of the integrated transgenes, a mixture of several independently transformed calli were used as starting material. Also the processing of the rRNA does not seem to suffer from the loss of the upstream SalI repeats. Therefore, it seems that sequences from the so-called "upstream Sal repeats" (see Fig 1) have no significant influence on the expression level of the ribosomal gene unit in freshly transformed callus material. Fig 7 shows a quantitative evaluation of the gene expression of ribosomal gene-units.

However, it was demonstrated that the DNA-fragment comprising the "upstream Sal repeats" has an influence on the stability of the introduced DNA. Fig. 8(a)-(e) shows constructs which were compared with each other (R4-R8 are E. coli-Agrobacterium shuttle vectors with RK2 origin of replication, whereby "Br" and "Bl" indicate the right, respectively left T-DNA border; (a) binary vector R4: comprises the complete ribosomal unit from A. thaliana next to a hygromycin resistance gene (HPT); (b) binary vector R5:

is similar to R4, without the "upstream Sal repeats" (lacks the DNA sequence of SEQ ID N° 1 from nucleotide 1269 to 3002); (c) binary vector R6: comprises only the intergenic region (IGR) of the ribosomal DNA next to the hygromycin resistance gene (was constructed by insertion of the DNA fragment having the sequence of SEQ ID N° 1 from 596 to 5373 into pBIB Hyg); (d) binary vector R7: is similar to R6 but lacks the "upstream Sal repeats"(deletion of the DNA sequence of SEQ ID N° 1 from nucleotide position 1269 to 3002); (e) binary vector R8: comprises only the RNA encoding part of the ribosomal DNA adjacent to a resistance gene (similar to R1 but without the sequence of SEQ ID N° 1 from nucleotide position 1269 to 5075)). The sizes of the plasmids are about (in base pairs) 23810 (R4), 21810 (R5), 16010 (R6), 14010 (R7) and 19960 (R8).

All constructs according to the invention cause a stabilization of the transgene, which can be observed as the presence of a higher copy number and/or higher transformation efficiency, particularly as a more stable expression of the structural gene, particularly a marker gene such as the hygromycin phosphotransferase genes).

A hypothesis to explain this effect, is that this sequence shields the ribosomal repeats from recombination and/or methylation. This sequence could also be responsible for the localization of the ribosomal DNA in a special subregion of the nucleus (the nucleolus). Since the experiments show that adjacent genes which are transcribed by RNA polymerase II tolerate the localization in the neighbourhood of a nucleolus, as well as demonstrate activity, it is concluded that the stabilizing effect is extended to this kind of genes.

Example 2: Comparison of constructs according to the invention (with "upstream Sal repeats", USR) with a traditional construct (without "upstream Sal repeats", USR) particularly concerning transgene copy number and expression level.

2.1. As described in the Experimental section, DNA was prepared from transformed callus material and digested with the restriction enzymes BamHI and HindIII. Both enzymes cut within the transferred DNA, thus all copies integrated in the genome will run as a single band upon size separation by gel electrophoresis. By Southern blotting using the hygromycin resistance gene as probe, the relative number of the transgene copies was determined in different calli by comparison of the intensity of the hybridizing bands with the help of a Phospho imager. As a control, the DNA blot was hybridized using as probe, a DNA known to be present in two copies in the genome (chlorata 42). The relative intensity of the bands determined as described above, was used to estimate the amount of DNA in the different lanes. From the comparison of the intensity of the bands of both probes, it was determined that the analysed transgenes were present in the following copy

number:

Comparison of construct Fig.8-A (with USR; according to the invention; for the time being, this construct is regarded by the applicant as the best mode for carrying out the invention) with a construct Fig. 8-B (without USR; state of the art):
construct with USR: copy number per haploid genome in 6 instances
7; 5.5; 0.5; 3.5; 3; 0.5 leading to an average of 3.3 $\pm$ 2.7 copies.

Construct without USR: copy number per haploid genome in 5 cases
2; 1; 2; 1.5; 1.5 leading to an average of 1.6 $\pm$ 0.5 copies.

Comparison of construct Fig.8-C (with USR; according to the invention) with a construct Fig. 8-D (without USR; state of the art):

construct with USR: copy number per haploid genome in 4 instances
1.5; 1; 2.5; 2; leading to an average of 1.8 $\pm$ 1.2 copies.

Construct without USR: copy number per haploid genome in 5 cases
2; 1; 1; 1.5; 0.5 leading to an average of 1.2 $\pm$ 0.5 copies.

It should also be remarked that the dispersion in constructs with USR is significantly higher. This allows the interpretation that the expression of trangenes is guaranteed to occur in a sufficient manner in a broader spectrum of transgene-configurations, e.g. also in quite high or quite low copy-number.

It was also demonstrated that with the constructs according to the invention, particularly when they are integrated in the genome in a good position, an augmentation of the copy number can be achieved (see results with construct 8-A).

2.2 Two DNA constructs were built, that express the reporter gene beta-glucuronidase (gus) under control of the well-characterized CaMV35S promoter. Adjacent to this gene again the USR sequence is either present or absent (see Fig. 9A and B).

The vector represented in Fig. 9B was constructed by introduction of a DNA fragment comprising the chimeric CaMV35S-gus gene from pRTgus (Topfer et al. (1993) "Expression vectors for high-level gene expression in dicotyledonous and monocotyledonous plants" Meth.Enzymol. 217, 66-78") in the polynucleotidelinker between the nos terminator and the hyg gene of pBIB Hyg (Becker et al. 1990). The vector represented in Fig. 9A was constructed by introduction of an EcoRI-SalI DNA fragment

having the DNA sequence of SEQ ID N° 1 from nucleotide position 596 to 2862, in the vector of Fig. 9B in such a way that the end of the ribosomal DNA fragment located originally adjacent to the PolI promoter is now located adjacent to the CaMV35S promoter.

The activity of the enzyme encoded by the transgene was estimated for transgenic callus material (2 to 25 mg) homogenized in 200 µl extraction buffer (50 mM sodium phosphate pH 7, 10 mM 2-mercapto-ethanol, 10 mM EDTA, 0.1 % SDS, 0.1 % Triton X-100). After centrifugation (10 min, 4°C, 18 000 rpm) the activity was measured. Therefore, para-nitrophenyl-beta-glucuronide (pNPG; 2mM) was added and incubated from 10 to 30 min at 37°C. The reaction was stopped by addition of 0.2 M sodium-carbonate (modified after Gallagher, ed., GUS protocols, Academic Press, 1992).

Comparison of construct with, respectively without USR (indicated are relative enzyme units per gram fresh weight transformed calli):

construct with USR: measured activities 2.3; 6.9; 11.2; 0.3; 1.2; 0.3; 1.0; 2.3; 2.0; average 3.1 ± 2.8.

Construct without USR: measured activities 0.1; 1.6; 0.0; 1.1; 0.6; 0.2; 0.6; 1.5; 0.8; average 0.7 ± 0.4.

It is remarkable that both the average level of activities is considerably higher with USR (difference factor 4) , and the dispersion of the activities is larger . Further with the constructs with USR no callus occurred which exhibited an extremely low level of activity (e.g. 0.0 or 0.1). This allows to conclude that a larger range of activity levels from the transgenes can be obtained in a stable way.

Also here was demonstrated that with integration of the constructs according to the invention on a certain place a higher expression level can be reached (see activities from 6.9 to 11.2 with a USR -construct according to Fig. 9A). This strongly enhanced activity could be due to the fact that the r-DNA creates a favourable chromatin-environment for transcription.

A further reason that a broader spectrum of trangene -configurations is stable and allows gene-expression, is apparently the fact that the r-DNA circumvents the normal control mechanisms for euchromatin, e.g. by suppressing pairing of homologous region, suppression of homologous recombination, suppression of the methylation occurring in euchromatin.

Also the activity from transgenes in the presence of certain sequences from the ribosomal DNA is guaranteed when several units are present in tandem repeat or on several places in the genome so that a higher copy number leads to higher expression.

Example 3: Analysis of corn plants transformed with DNA constructs according to the invention.

The rRNA intergenic region of corn is amplified by PCR using oligonucleotides corresponding to the 3' end of the conserved 25S mature rRNA encoding region and the 5' end of the conserved 18S rRNA mature rRNA encoding region (using the sequence available from EMBL database under the accession number EMBL X03990). The oligonucleotides are designed to include suitable restriction sites at the extremities of the amplified fragment.

The fragment is cloned upstream of a CaMV35S promoter region operably linked to a region encoding phospinotricinacetyl transferase (PAT encoded by bar) as described in WO 92/09696 in such orientation that the intergenic region sequence proximal to the mature 18S rRNA coding region is now proximal to the chimeric bar gene. The DNA construct comprising the intergenic rDNA and the chimeric bar gene are inserted between the borders of the T-DNA vector pGSV5 (described in W097/13865).

The DNA-construct is then integrated into the nuclear genome of corn according to the methods described in WO92/09696 or EP 0469273.

Transgenic corn lines are analysed for expression level (by determination of the level of PAT activity) and the copy number of transgenes (by Southern hybridizations). Both the average copy number and average expression level of the chimeric bar gene are higher in the lines transformed by the DNA constructs comprising the intergenic repeat, than in control lines transformed by DNA constructs without intergenic repeat.

Example 4: Analysis of oilseed rape plants transformed with DNA constructs according to the invention.

The rRNA intergenic region of oilseed rape is amplified by PCR using oligonucleotides corresponding to the 3' end of the conserved 25S mature rRNA encoding region and the 5' end of the conserved 18S rRNA mature rRNA encoding region (using the sequence of a related species such as the one available from EMBL database under the accession number

X60324). The oligonucleotides are designed to include suitable restriction sites at the extremities of the amplified fragment.

The fragment is cloned upstream of a CaMV35S promoter region operably linked to a region encoding phospinotricinacetyl transferase (PAT encoded by bar) as described in WO 92/09696 in such orientation that the intergenic region sequence proximal to the mature 18S rRNA coding region is now proximal to the chimeric bar gene. The DNA construct comprising the intergenic rDNA and the chimeric bar gene are inserted between the borders of the T-DNA vector pGSV5 (described in W097/13865).

The DNA-construct is then integrated into the nuclear genome of oilseed rape according to the methods described in WO97/13865.

Transgenic oilseed rape lines are analysed for expression level (by determination of the level of PAT activity) and the copy number of transgenes (by Southern hybridizations). Both the average copy number and average expression level of the chimeric bar gene are higher in the lines transformed by the DNA constructs comprising the intergenic repeat, than in control lines transformed by DNA constructs without intergenic repeat.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Plant Genetic Systems N.V.
(B) STREET: Jozef Plateaustraat 22
(C) CITY: Gent
(E) COUNTRY: Belgium
(F) POSTAL CODE (ZIP): B-9000
(G) TELEPHONE: 32-9-2358454
(H) TELEFAX: 32-9-2231923

(ii) TITLE OF INVENTION: DNA-constructs and methods to produce proteins using these DNA-constructs

(iii) NUMBER OF SEQUENCES: 6

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5373 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Arabidopsis thaliana

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:1..485
(D) OTHER INFORMATION:/note= "25S rDNA 3' end"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:486..5211
(D) OTHER INFORMATION:/note= "intergenic region"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:1263..1557
(D) OTHER INFORMATION:/note= "SalI box 1"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:1883..2177
(D) OTHER INFORMATION:/note= "SalI box 2"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:2503..3003
(D) OTHER INFORMATION:/note= "SalI box 3"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:5212..5373
(D) OTHER INFORMATION:/note= "18S rDNA 5' end"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GAATTCACCA AGTGTTGGAT TGTTCACCCA CCAATAGGGA ACGTGAGCTG GGTTTAGACC 60

GTCGTGAGAC AGGTTAGTTT TACCCTACTG ATGCCCGCGT CGCGATAGTA ATTCAACCTA 120

GTACGAGAGG AACCGTTGAT TCGCACAATT GGTCATCGCG CTTGGTTGAA AAGCCAGTGG 180

CGCGAAGCTA CCGTGCGCTG GATTATGACT GAACGCCTCT AAGTCAGAAT CCGGGCTAGA 240

AGCGACGCAT GCGCCCGCCG CCCGATTGCC GACCCTCAGT AGGAGCTTAG GCTCCAAAGG 300

CACGTGTCGT TGGCTAAGTC CGTTCGGCGG AACGGTCGTT CGGACCGCCT TGAATTATAA 360

TTACCACCGA GCGGCGGGTA GAATCCTTTG CAGACGACTT AAATACGCGA CGGGGTATTG 420

TAAGTGGCAG AGTGGCCTTG CTGCCACGAT CCACTGAGAT TCAGCCCTTT GTCGCTAAGA 480

TTCGACCCTC CCCTAAATCA CTCCAAAAAA AACAATCCCC AATTCTACAC AAGTGTTTCT 540

ACACTAACAA AGCAACAGCT CCTTAACGAA TTCCCAACTT TACACGAGCT CGTCTCTCGA 600

GGTTAAATGT TATTACTTGG TAAGATTCCG GACCTCGCCA AGTGTTTTGA AAACCCGCAA 660

CGCTCGCAAA GGTGGATAGT GAGAATAATA AGTGAAGAGA CAGACTTGTC CAAAACGCCC 720

ACCACGAAGG TGCATAGTGA GAAGAGTAAG TCAAGAGATA GACTTGTCCA AAAAGAAACG 780

GAAGAGAAAG CGTGGGGAGA CGCTCACGAA GGTGCATAGT GAGAAGAGTA AGTCAAGAGA 840

CAGACTTGTT CGAAAAGAAA CAGAAGAGAA TGCTTGGGGT TACACTCACG AAGGTGCATA 900

GTGAGAAGAG TAAGTCAAGA GACAGACTTG TTCGAAAAGA AACAAAAGAG AATGCTTGGG 960

GAGATAGAAG TGTGAGATAG TTCTCAAGCT AAGAAAGTTG TAAAAGCTAA GAACTAGCAT 1020

CAAATGATGG ATGAAACACA AGGTAGTTGT TGAAAAGTCA AACACTTGGT GATATGAACA 1080

CAAACGTTCA ATATGACAAA CCCATGCCAA GTAAAGAGAA AATGAAAACT GGTGATTGTT 1140

GCGGAAATCG TCCAGGATTC CTCGACCAGG ACTTGAAATC GTCGAGGGGA AAAAATCGGT 1200

TCCGAGGAAT CGTCGATCCG GACTTGGAAT CGTCGAGAAA AGTTTACCGG GTCCGAGGAT 1260

TTGTCGACCA GGAGTGGAAA TCGTCGAGAA AAATCTATCG GGTCCGAGGA ATCGTCGACC 1320

AGGACGAGGA ATCGTCGACC GGGTCCGAGG ATTTGTCGAC CAGGGGTTGA AATCGTCGAC 1380

CAGGTCCGAG ACTTCATCGA CCGGGTCCGA GGATTCGTCG ACCAGGAGTG GAAATCGTCG 1440

AGAAAAATCT ATCGGGTCCG AGGAATCGTC GACCAGGACG AGGAATCGTC GACCGGGTCC 1500

GAGGATTTGT CGACCAGGTC CGAGACTTCA TCGACCGGGT CCGAGGATTC GTCGACCAGG 1560

TCCGAGACTT CATCGACCGG GTCCAAGTAT TTGATTTTAT TTTGGAACCG GTGTCTCCTC 1620

AGACATTTCA ATGTATGTTG GTGCCAAGAG GGAAAAGGGC TATAAAGCTA TATAGGGGTG 1680

GGTGTGTGTA GTGGGAATTT TGCCCGCACG CGCGCGCGCG AATAAGAATA AGAATAAGTA 1740

ATAAGAATAA GAATAAGAAA AAGAAAAAAA AAAAAAAAAA AAAAAAACAA AAACTCCATA 1800
GAGTTGTTTT CTCAATCGGG TCCGAGGAAT CGTCGATCTG GACTTGGAAT CGTCGAGAAA 1860

AGTTTACCGG GTCCGAAAAT TTGTCGACCA GGAGTGGAAA TCGTCGAGAA AAATCTATCG 1920

GGTCCGAGGA ATCGTCGACC AGGACGAGGA ATCGTCGACC GGGTCCGAGG ATTTGTCGAC 1980

CAGGGGTTGA AATAGTCGAC CAGGTCCGAG ACTTCATCGA CCGGGTCCGA GGAATCGTCG 2040

ACCAGGAGTG GAAATCGTCG AGAAAAATCT ATCGGGTCCG AGGAATCGTC GACCAGGACG 2100

AGGAATCGTC GACCGGGTCC GAGGATTTGT CGACCAGGTC CGAGACTTCA TCGACCGGGT 2160

CCGAGGATTC GTCGACCAGG TCCGAGACTT CATCGACCGG GTCCAAGTAT TTGATTTTAT 2220

TTTGGAACCG GTGTCTCCTC AGACATTTCA ATGTATGTTG GTGCCAAGAG GGAAAAGGGC 2280

TATAAAGCTA TATAGGGGTG GGTGTGTGTA GTGGGAATTT TGCCCGCACG CGCGCGCGCG 2340

ATAAGAATAA GAATAAGTAA TAAGAATAAG AATAAGAAAA AGAAAAAAAA AAAAAAAAAA 2400

ACAAAAACAA AAACTCCATA GAGTTGTTTT CTCAATCGGG TCCGAGGAAT CGTCGATCTG 2460

GACTTGGAAT CGTCGAGAAA AGTTTACCGG GTCCGAAAAT TTGTCGACCA GGAGTGGAAA 2520

TCGTCGAGAA AAATCTATCG GGTCCGAGGA ATCGTCGACC AGGACGAGGA ATCGTCGACC 2580

GGGTCCGAGG ATTTGTCGAC CAGGGGTTGA AATCGTCGAC CAGGTCCGAG ACTTCATCGA 2640

CCGGGTCCGA GGAATCGTCG ACCAGGACGA TGAATGGTCG ATGAAAATCT ATCGGGTTCG 2700

AGGAATGGTC GACCAGGGGT TGAAATCGTC GACCAGGTCC GAGACTTCAT CGACCGTGTC 2760

CGAGGAGTGG TCGAGGGTTT GTCGACCAGG ACGAGGAATC GTCGACCGGG TCCGAGGATT 2820

TGTCGACCAG GGGTTGAAAT CGTCGACCAG GACCGAGAAT TCGTCGACCA GGACGGCGGA 2880

ACCCTCGACC AGGACGATGA ATGGGCGATG AAAATCTATC GGGTTCGAGG AATGGTCGAC 2940

CAGGGGTTGA AATCGTCGAC CAGGTCCGAG ACTTCATCGA CCGGGTCCGA GGATTCGTCG 3000

ACCAGGACGG CCGGATGTCC GAGAAAAAAA AATGTTGCCG AATAACTTTC GAAAATCATT 3060

GGATATGATG CAATGTTTTG TGATCGAATC TCTTAAAATA CATCAATAAA GAGTTTAGGA 3120

TGTCAAGTTT GCATCAAATA TGCCCACGGA GCCCCAACTA GACCATGAAA ATCCGATGTT 3180

GTATCAGGTC AAATGACCTA GCTAGAGGTG TCAAAAAATT ATGAAAATTT ACCAGAAAAT 3240

AGGATTTAGT ATCCTTATGA TGCATGCCAA AAAGAATTTT CAAATTCCAA GTATTTCTTT 3300

TTTCTTGGCA CCGGTGTCTC CTCAGACATT TCAATGTCTG TTGGTGCCAA GAGGGAAAAG 3360

GGCTATTAAG CTATATAGGG GGGTGGGTGT TGAGGGAGTC TGGGCAGTCC GTGGGGAACC 3420

CCCTTTTTCG GTTCGGACTT GGGTAGCGAT CGAGGGATGG TATCGGATAT CGGCACGAGG 3480

AATGACCGAC CGTCCGGCCG CCGGGATTTT CGCCGGAAAA CTTTTCCGGG CACTTTTCCG 3540

GCGATCGGTT TTGTTGCCTT TTTCCGAGTT TTCTCAGCAG TTCTCGGACA AAAACTGCTG 3600

AATCGTCGAG GAGAATGGGC TTGCTTGCGT GGGCTGCCAT TAGTTCTTCG AGGCGTTAGG 3660

GTGGCGGCGG TATAAAAGTG TCGGAGTTTT TTCAGCAGTT CTCGGACAAA AATTGCTGAG 3720

TGGCCGAGAA GAATGGGCGT GTCATGCGTG GGCTGACATG GATTCTTCGA GGCCTAGGGG 3780

TGGCGGTATA TAACTTGTTC GCATGATATT ACCGAGATGT CCCCACGGGC ATCTTTTCAC 3840

CTCGTCGCCG AAGAGAATGG GCGTGTCATG GCATGGGCTG ACATGGATTC TCCTAGGCCG 3900

TTTGGGTGGC GGTATAGTCG TCTTGCGCAC GAAATACCGA GATGTCCCCA TGGGCATCGA 3960

TTCCACCCGC CTAGGTTGGA TGGGCGTGCT TCGTCGGAAA GCATGGATCC GCCTAGGCTG 4020

TCCCGAGTGT GAGCGAGGTG TGAGTGTCGC CCATGGGCAT CGACACCTTG CGGCTAGGAA 4080

CTGGAACGAG ACGGGTGGCA AAGATTTCGA GTAGCACTTC ATACTACCGT GGGTTTTTTA 4140

AACCTTCCGA GTTTTGTTGA TGTTATTCCG AGAATTAGCA AACCGTAACG AAGATGTTCT 4200

TGGCAACCAT CTTTTGATGG GAGTCCGGCT GTTCGATAGC CGGCCAAGGG TGATGAACGA 4260

AATGTGAACC CTTGTCTCGC CTAGGTTGGA TGGGCGTGCT TCGTTGGAAA GCATGGATCC 4320

GCCTAGGCTG TCCCGAGTGT GAGCGAGGTG TGAGTGTCGC CCATGGGCAT CGACACCTTG 4380

CGGCTAGGAA CTGGAACGAG ACGGGTAGCA AAGATTTCGA GTAGCACTTC ATACTACCGT 4440

GGGTTTTTTA AACCTTCCTA GTTTTGTTGA TGTTATTCCG AGAATTAGCA AACCGTAACG 4500

AAGATGTTCT TGGCAACCAT CTTTTGATGG GAGTCCGGCT GTTCGAAAGC CGGCCAAGGG 4560

TGATGAACGA AATGTGAACC CTTGTCTCGC CTAGGTTGGA TGGGCGTGCT TCGTTGGAAA 4620

GCATGGATCC GCCTAGGCTG TCCCGAAGTA TCTCGCGCTT GTACGGCTTT GGCTCGGATT 4680

CGTCCGTCTT CTTTCTTCTT AGCCGAGTAC TTCGGTAGAT TAGTTGGAAC GATTGATGAT 4740

TTTGAGTTAA TTGAACGTTC GGCGTATGAG TGGTGATCGG ATAGCTAGTG TTCGTAGGCT 4800

CCATGCTCGC GCATCGAACT ACCTACCACC TATCCTTCTC AGTTAATTCA CGGGCGATGT 4860

TACGCTCGAT GATGAGTTCC GGGGCCTGTG TTTCGTACCT AATTTGAAGG AATTGTTGAG 4920

TTTGGTTTAC ACCTTTGCCC GCGGCTTCTC CTTCGTGGGG AAGTCGTGGG CTCAAACATC 4980

GGCGCTTGTT CACCTCTCGT CATCGCATTT GTTGCCTTGC TCGCATTGGT GAATGAGTTG 5040

CGGGTTGAAA TCTCGGATGC GGAAAAGTTG TCGACGGTGA CTCGAAGTGA TTCAGTCCCG 5100

CCAAAGCTCA TCCGTCCTTC GGGCAAAAGA TGACGGTCAA GACCTCGTCC TTTCTCTCTT 5160

TCCATTGCGT TTGAGAGGAT GTGGCGGGGA ATTGCCGTGA TCGATGAATG CTACCTGGTT 5220

GATCCTGCCA GTAGTCATAT GCTTGTCTCA AAGATTAAGC CATGCATGTG TAAGTATGAA 5280

CGAATTCAGA CTGTGAAACT GCGAATGGCT CATTAAATCA GTTATAGTTT GTTTGATGGT 5340

AACTACTACT CGGATAACCG TAGTAATTCT AGA 5373

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:
CCAAGGTAAC CTTCGACGT 19

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CGAAGGTTAC CTTGGACGT 19

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "oligonucleotide E"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GAAGACGTCG AAGGTTACCT TGG 23

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "oligonucleotide Q"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CCCGGTTCGC TCGCCGTTAC TAAG 24

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 236 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "5'end of the A. thaliana 25S rDNA"

(ix) FEATURE:

(A) NAME/KEY: -

(B) LOCATION:1..10

(D) OTHER INFORMATION:/note= "3' end of the 5SrDNA"

(ix) FEATURE:

(A) NAME/KEY: -

(B) LOCATION:198..236

(D) OTHER INFORMATION:/note= "3' end of the 25S rDNA"

(ix) FEATURE:

(A) NAME/KEY: -

(B) LOCATION:220

(D) OTHER INFORMATION:/note= "previously assumed 5'end of

the 25S rDNA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GGTGTCACAA ATCGTCGTCC CTCACCATCC TTTGCTGATG CGGGACGGAA GCTGGTCTCC 60

CGTGTGTTAC CGCACGCGTT GGCCTAAATC CGAGCCAAGG ACGCCTGGAG CGTACCGACA 120

TGCGGTGGTG AACTTGATCC ATTACATTTT ATCGGTCGCT CTTGTCCGGA AGCTGTAGAT 180

GACCCAAAGT CCATATAGCG ACCCCAGGTC AGGCGGGATT ACCCGCTGAG TTTAAG 236

Claims

1. A DNA construct, comprising the following operably linked DNA fragments:
- a DNA fragment comprising a ribosomal DNA sequence, preferably derived from a plant;
- a fragment comprising an expressible promoter region, especially a plant-expressible promoter region;
- a heterologous coding region; and optionally
- a transcription termination and polyadenylation region, preferably a region which is active in plant cells.

2. A DNA-construct according to claim 1, wherein said plant-expressible promoter region is a promoter recognized by RNA polymerase II.

3. A DNA-construct according to claim 1, wherein said ribosomal DNA comprises the promoter.

4. A DNA-construct according to any one of claims 1 to 3, wherein said ribosomal DNA sequence is derived from the plant comprising the DNA-construct.

5. A DNA-construct according to any one of claims 1 to 4, wherein said ribosomal DNA sequence is derived from the intergenic region of the ribosomal DNA.

6. A DNA construct according to claim 5, wherein said ribosomal DNA sequence comprises the upstream SalI repeats from the intergenic region from the ribosomal DNA of Arabidopsis thaliana or a similar region from another plant.

7. A DNA construct according to any one of claims 1 to 4, wherein said ribosomal DNA sequence comprises a DNA sequence selected from the DNA sequence of SEQ ID N° 1 from nucleotide position 486 to 5212, the DNA sequence of SEQ ID N° 1 from nucleotide position 1263 to nucleotide position 3003, the DNA sequence of SEQ ID N° 1 from nucleotide position 569 to nucleotide position 2862, the DNA sequence of SEQ ID N° 1 from nucleotide position 1263 to nucleotide position 2862, the DNA sequence of SEQ ID N° 1 from nucleotide position 486 to 5212, the DNA sequence of SEQ ID N° 1 from nucleotide position or 596 to 5373.

8. A DNA-construct according to any one of claims 1 to 7, wherein said heterologous coding region encodes a vaccine, an antibody, a therapeutical protein, an

insecticidal protein such as a Bt toxin or the minimal toxic fragment thereof, a protein used in food technology, an antisense-RNA or a ribozyme.

9. A DNA construct according to claim 1, wherein the DNA construct is comprised within a T-DNA transformation vector.

10. A method to produce proteins, comprising the following steps:
- introducing a DNA-construct of any one of claims 1 to 9 in a suitable host organism;
- cultivating the host-organism under conditions which allow expression of the protein encoded by the heterologous coding region; and
- harvesting the expressed protein.

11. A method for enhancing the stability, the copy number or the expression of a transgene, especially in a plant, comprising the following steps:
- introducing a DNA construct of any one of claims 1 to 9 in a cell, preferably in a plant cell; and
- regenerating an organism, preferably a plant, from the transformed cell.

12. A method according to claim 11, wherein said plant is selected from Arabidopsis thaliana, tobacco, corn, wheat, potato, rice, soy beans, barley, rye, a Brassica species, a Beta species or manioc.

13. A cell, preferably a plant cell, comprising the DNA-constructs of any one of claims 1 to 9, integrated in the nuclear genome.

14. A plant cell according to claim 13, wherein said plant cell is derived from Arabidopsis thaliana, tobacco, corn, wheat, potato, rice, soy beans, barley, rye, a Brassica vegetable, a Beta species or manioc.

15. A plant comprising the plant cells of claim 13.

16. The use of a DNA fragment comprising the intergenic region of a ribosomal DNA of a plant, to enhance stability, the copy number or the expression of a transgene in a plant.

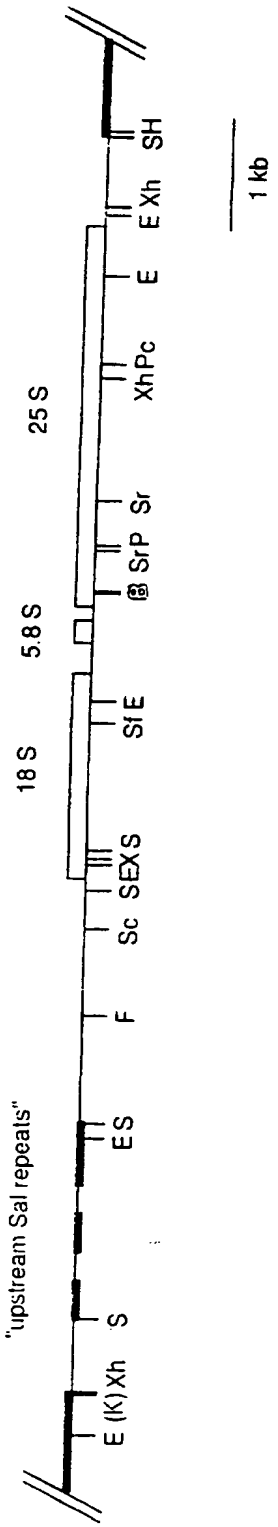
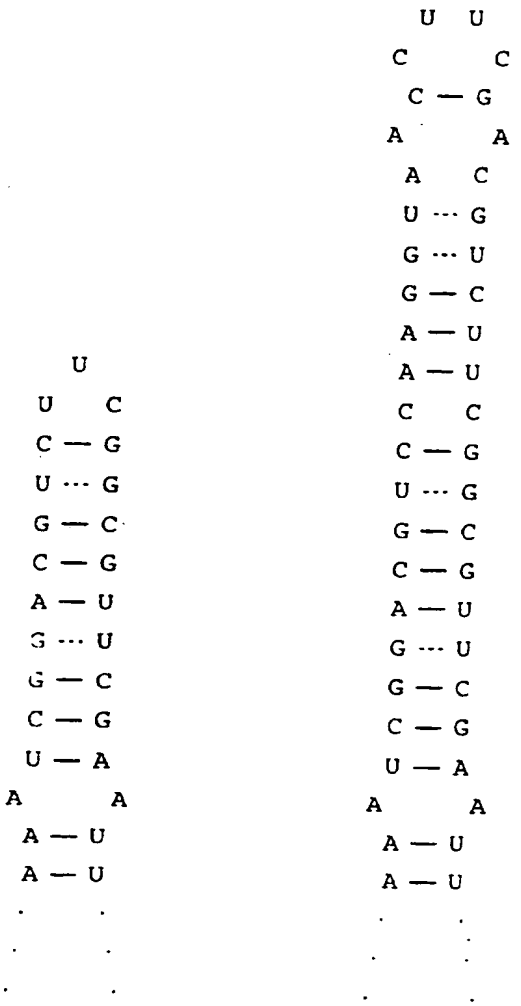

"upstream Sal repeats"
E (K) Xh
S
E S
F
Sc
S E X S
Sf E
18 S
5.8 S
25 S
Sr P
Sr
Xh Pc
E
E Xh
SH
1 kb

Fig.1

(a)



(b)

G A A G A C G T C G A A G G T T A C C T T G G

Fig.2

Fig.3

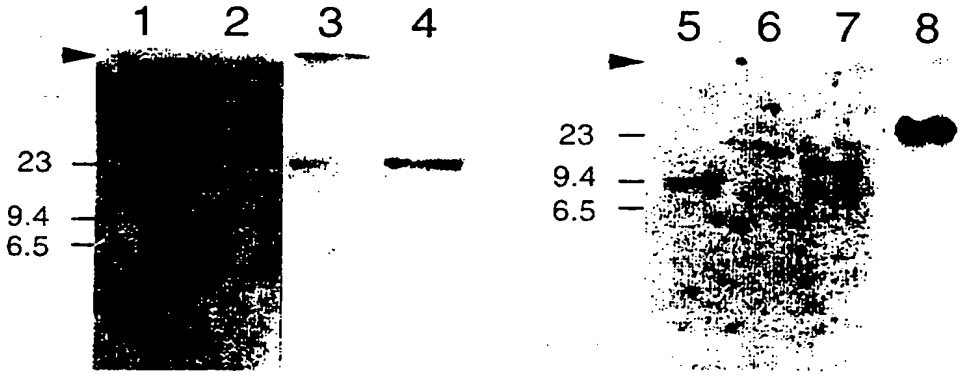


Fig.4

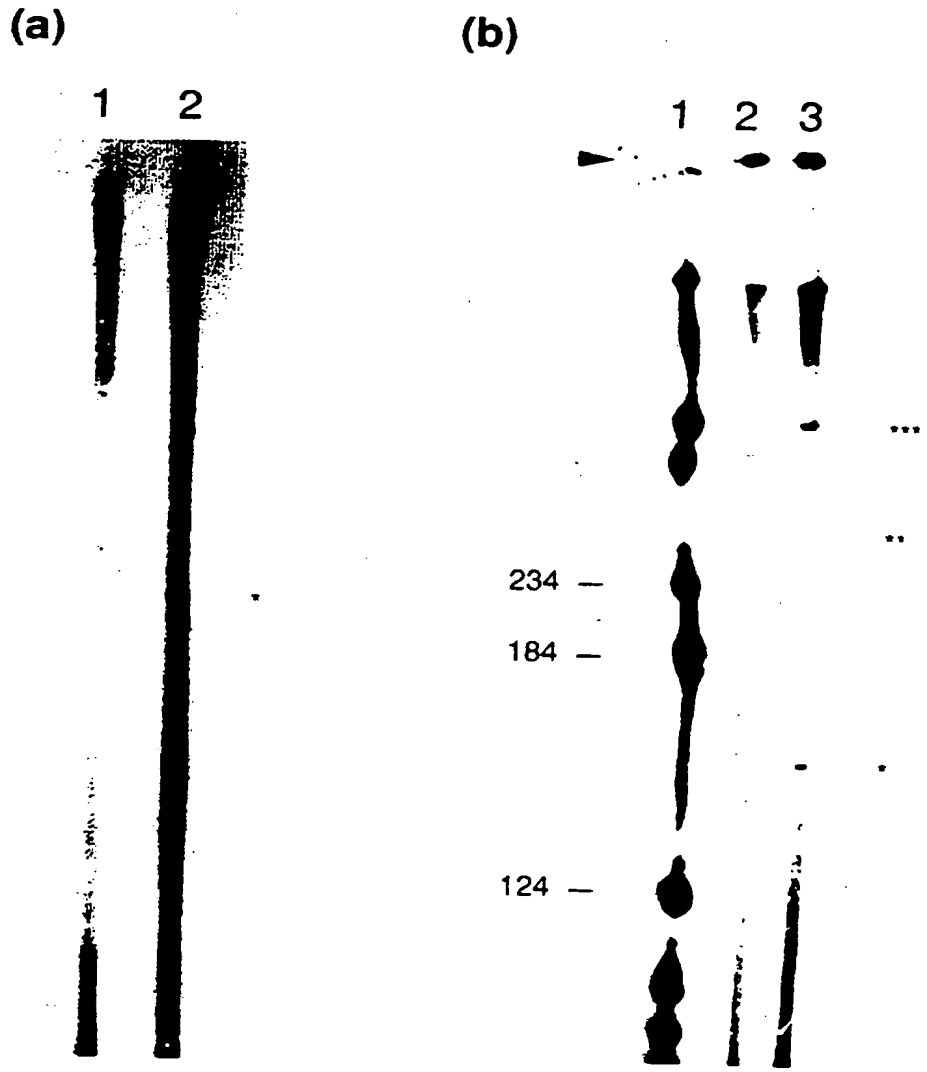


Fig.5

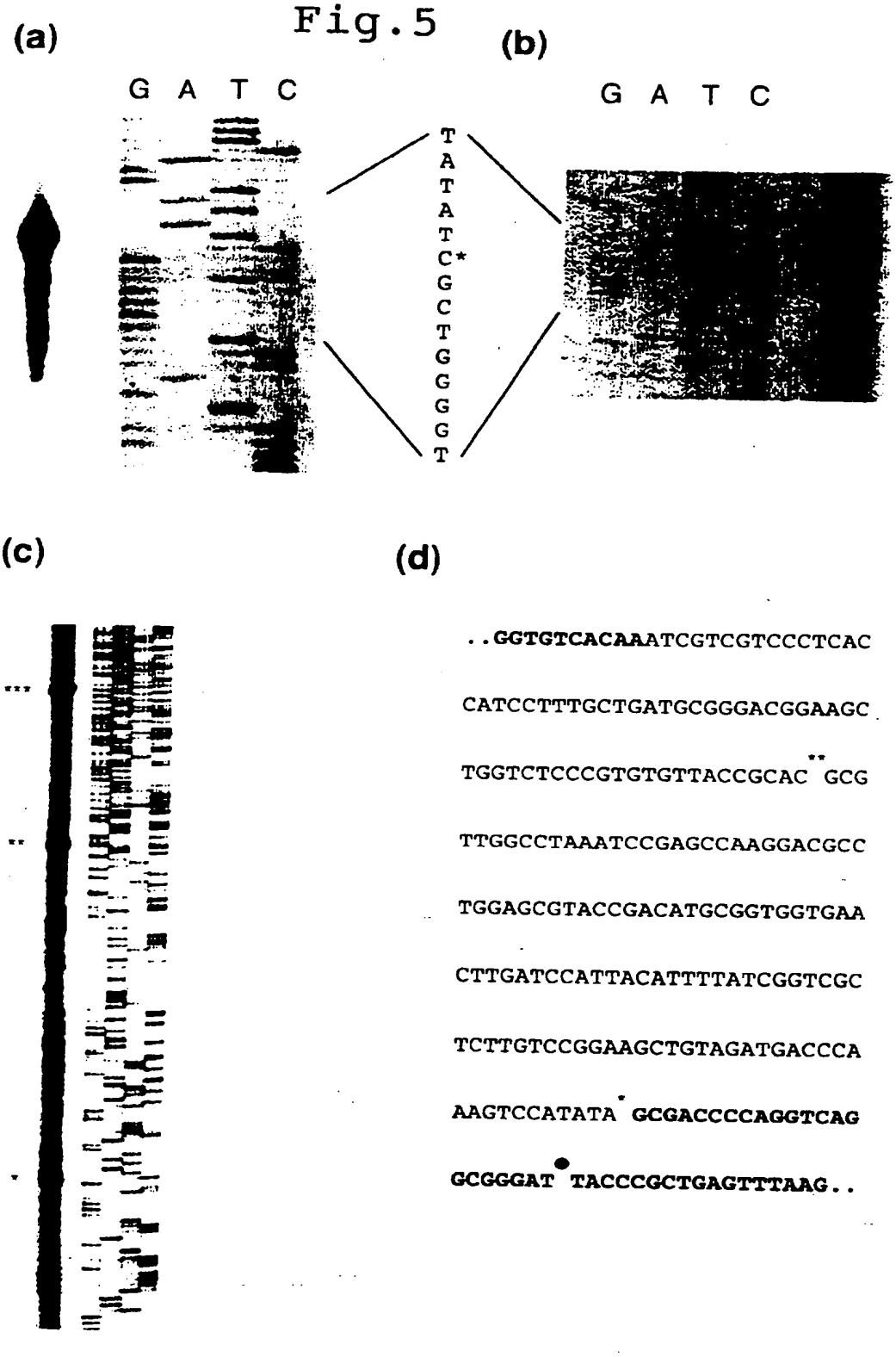

(a)
G A T C
(b)
G A T C
T A T A T C* G C T G G G G T
(c)
(d)
..GGTGTCACAAATCGTCGTCCCTCAC
CATCCTTTGCTGATGCGGGACGGAAGC
TGGTCTCCCGTGTGTTACCGCAC GCG
TTGGCCTAAATCCGAGCCAAGGACGCC
TGGAGCGTACCGACATGCGGTGGTGAA
CTTGATCCATTACATTTTATCGGTCGC
TCTTGTCCGGAAGCTGTAGATGACCCA
AAGTCCATATA GCGACCCCAGGTCAG
GCGGGAT TACCCGCTGAGTTTAAG..

Fig.6

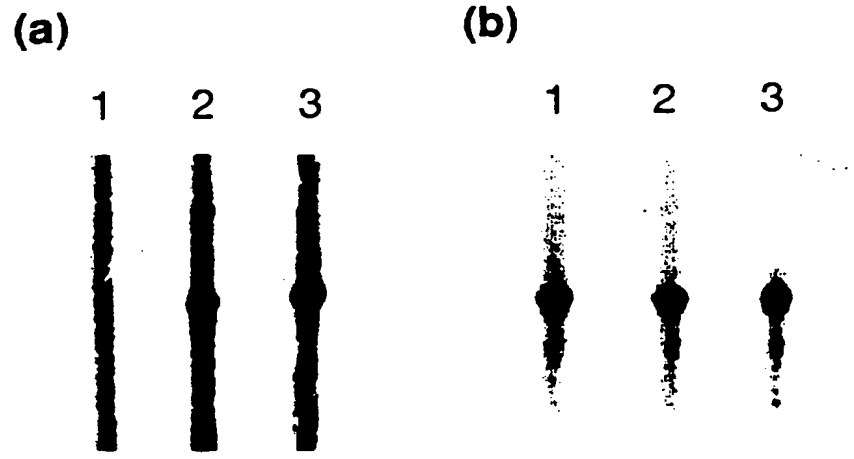


Fig.7

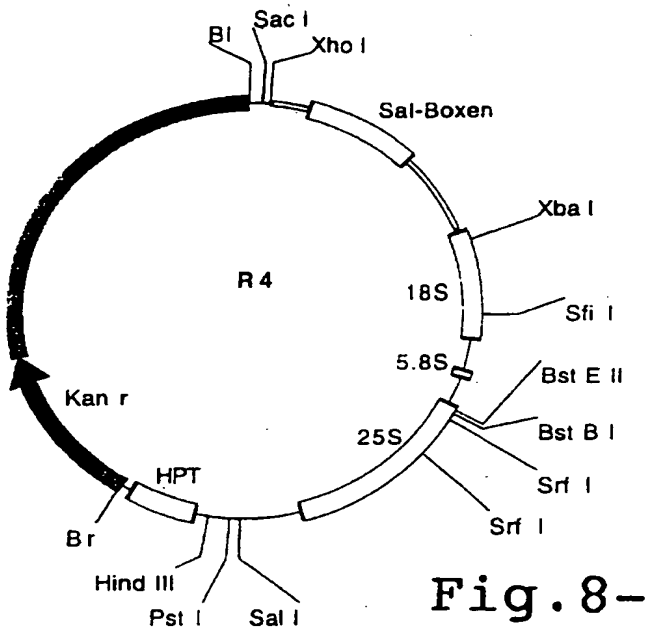


Fig.8-A

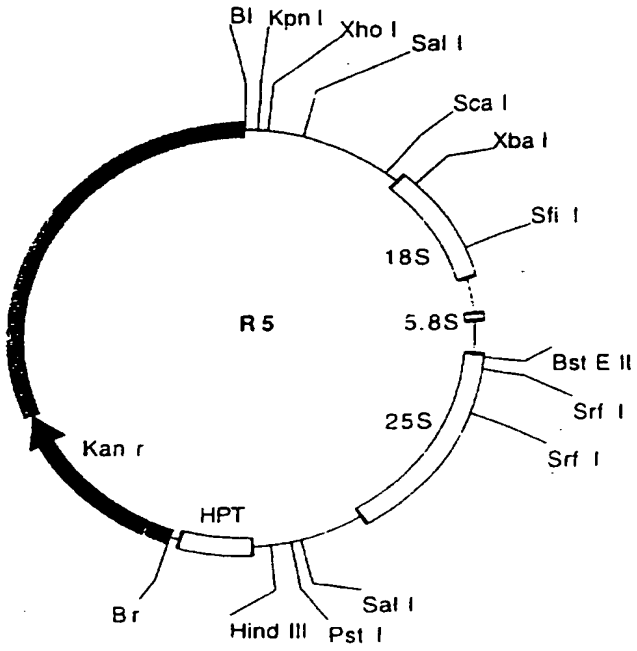


Fig.8-B

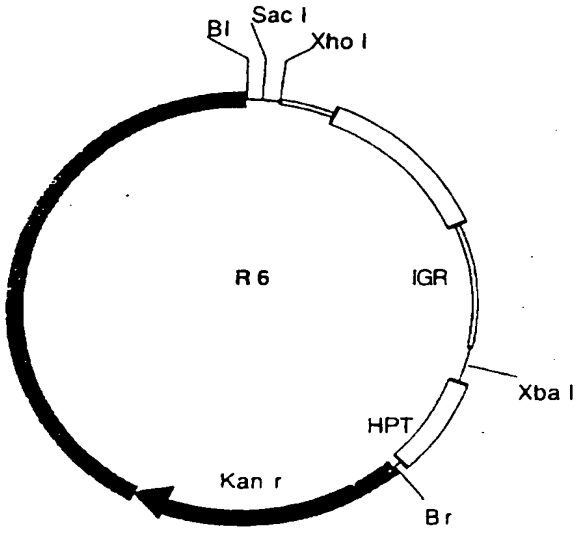


Fig.8-C

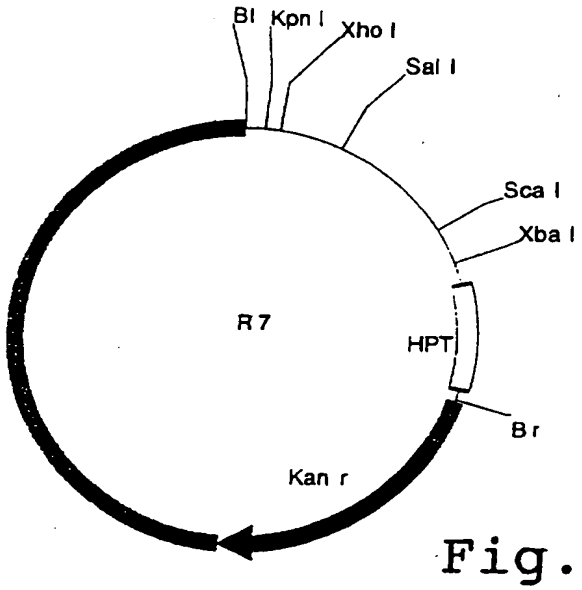

Bl
Kpn I
Xho I
Sal I
Sca I
Xba I
R7
HPT
Br
Kan r

Fig.8-D

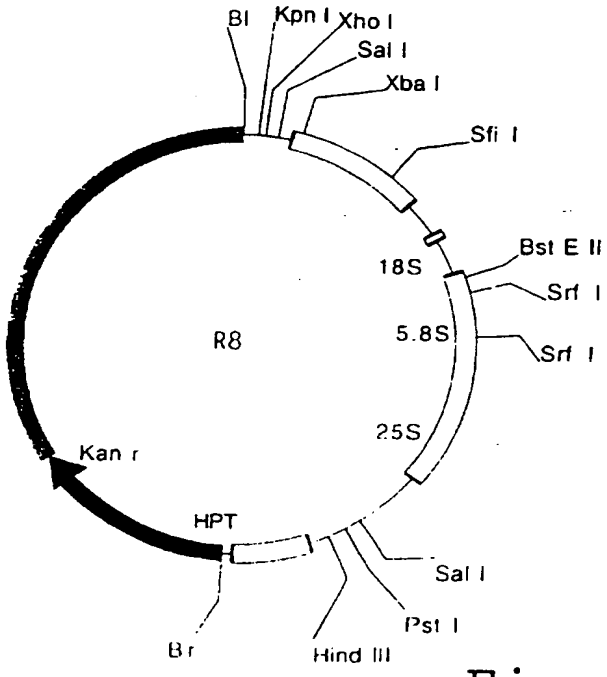
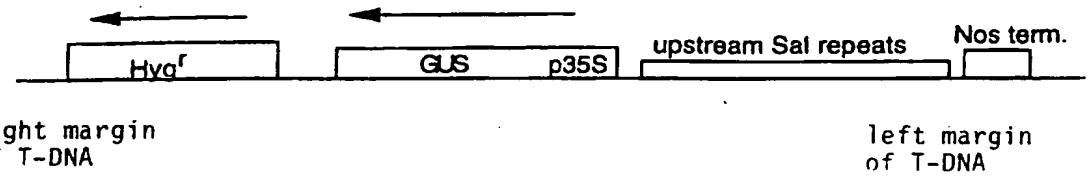

Bl
Kpn I
Xho I
Sal I
Xba I
Sfi I
Bst E II
18S
Srf I
R8
5.8S
Srf I
25S
Kan r
HPT
Sal I
Pst I
Br
Hind III

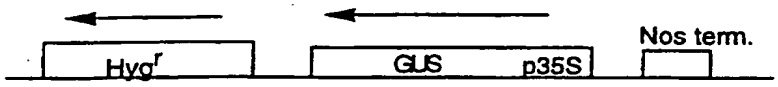
Fig.8-E



right margin of T-DNA

left margin of T-DNA

Fig. 9-A



right margin of T-DNA

left margin of T-DNA

Fig. 9-B

INTERNATIONAL SEARCH REPORT

Inter. onal Application No
PCT/EP 97/05217

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C12N5/10 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 91 00920 A (UNILEVER PLC ;UNILEVER NV (NL)) 24 January 1991 * see esp. p. 3 l. 24-36 * ---	1,8,10, 11
X	WO 86 00089 A (CIBA GEIGY AG) 3 January 1986 * see the whole document, esp. ex. 9 * --- -/--	1,2, 8-10, 13-15

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
17 February 1998	27/02/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Kania, T

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte. .onal Application No
PCT/EP 97/05217

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No
X	DOELLING J. ET AL.: "Functional analysis of Arabidopsis thaliana rRNA gene and spacer promoters in vivo and by transient expression" PNAS,U.S.A., vol. 90, no. 16, 15 August 1993, pages 7528-7532, XP002055921 cited in the application	1,3,5-7, 11,13
A	* see the whole document, esp. p. 7531 l. 17-20, p.7532 *	16
A	DOELLING J. AND PIKAARD C.: "The minimal ribosomal RNA gene promoter of Arabidopsis thaliana includes a critical element at the transcription initiation site" THE PLANT JOURNAL, vol. 8, no. 5, November 1995, pages 683-692, XP002055922 cited in the application see the whole document	1-16
P,X	WANZENBÖCK E. ET AL.: "Ribosomal transcription units integrated via T-DNA transformation associate with the nucleolus and do not require upstream repeat sequences for activity in Arabidopsis thaliana" THE PLANT JOURNAL, vol. 11, no. 5, May 1997, pages 1007-1016, XP002055923 see the whole document	1,3-7,9, 13-15

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter onal Application No
PCT/EP 97/05217

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9100920 A	24-01-91	AT 160176 T	15-11-97
		CA 2063592 A	08-01-91
		DE 69031710 D	18-12-97
		EP 0481008 A	22-04-92
		EP 0778348 A	11-06-97
		JP 5501949 T	15-04-93
WO 8600089 A	03-01-86	DK 70486 A	13-02-86
		EP 0182838 A	04-06-86
		JP 61502377 T	23-10-86